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Metabolic Regulation of Terpenoid Biosynthesis in Rhodospirillum Rubrum

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Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
Metabolic Regulation of Terpenoid Biosynthesis in Rhodospirillum rubrum

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Department Chemistry

Abstract
Rhodospirillum rubrum is a bacterium that has the metabolic versatility to produce energy through four different pathways: fermentation, photosynthesis, nitrogen-fixation and cellular respiration.1 R. rubrum can synthesize terpenoids that are utilized in energy production and other cellular functions.2 Different terpenoids are produced depending on what energy the bacteria's environment provides. This is an indication that the biosynthetic gene expression differs in different metabolic conditions. The goal of this experiment was to investigate the function and expression of the terpenoid biosynthetic gene A2950. Understanding the metabolic process of R. rubrum will assist in improving the efficiency of modern-day cyanobacteria and potentially lower the cost of engineered molecules.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature _____ Date _____

Faculty Sponsor Signature _____ Date _____

Metabolic Regulation of Terpenoid Biosynthesis in *Rhodospirillum rubrum*

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ABSTRACT

Rhodospirillum rubrum is a bacterium that has the metabolic versatility to produce energy through four different pathways: fermentation, photosynthesis, nitrogen-fixation and cellular respiration.[1] *R. rubrum* can synthesize terpenoids that are utilized in energy production and other cellular functions.[2] Different terpenoids are produced depending on what energy the bacteria's environment provides. This is an indication that the biosynthetic gene expression differs in different metabolic conditions. The goal of this experiment was to investigate the function and expression of the terpenoid biosynthetic gene A2950. Understanding the metabolic process of *R. rubrum* will assist in improving the efficiency of modern-day cyanobacteria and potentially lower the cost of engineered molecules.

INTRODUCTION

Only four terpenoid biosynthetic genes exist in the *R. rubrum* genome.[4] Three genes, A2618, A2950 and A2983, encode for putative prenyl-transferases that combine a 5- carbon substrate to an allylic substrate.[4] The fourth gene, RrSHC is a putative cyclase that likely produces cyclic products, hopanoids and diterpenoids. Preliminary results reveal that A2618 is an FPP synthase and A2983 is a GGPP synthase.[4] Although much has been learned about the metabolic versatility of terpenoid synthesis in *Rhodospirillum rubrum*, the function of the enzyme A2950 and the expression of *R. rubrum's* genes in aerobic and anaerobic environments remains poorly understood. Once the function is better understood, the regulation of the four terpenoid biosynthetic genes can be examined by expression analysis. The gene expression may be investigated by RT-PCR, which is capable of detecting and quantifying mRNA.

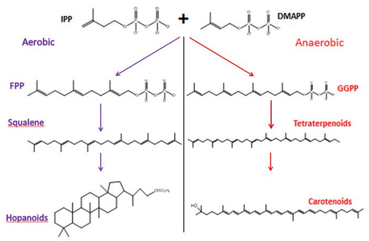

Figure 1. The pathway shown above illustrates the significant efficiency of *Rhodospirillum rubrum.* The bacterium has four terpenoid synthases.

METHODS

R. rubrum cultures were grown in 50 mL of media in 250 mL flasks anaerobically and aerobically at 30 ˚C. Genomic DNA was purified and cloned via PCR using the appropriate primers for gene A2950 and KAPA HiFI HotStart DNA polymerase. Gel electrophoresis was performed using the PCR products and the corresponding bands of A2950 were cut out and purified by gel purification using a Montage DNA Gel Extraction Kit (Millipore Corp., Billerica, MA, USA). The purified PCR products were cloned into the Gateway system vector pENTR/SD/D-TOPO (Invitrogen, Carlsbad, CA, USA) and inserted into a plasmid. A bacterial transformation was performed to insert the plasmids containing A2950 into *Escherichia coli* Top10 cells (Invitrogen, Carlsbad, CA, USA). The DNA that contains the genes of interest was recombined into pDEST/14 and pDEST/17 expression vectors using a LR Clonase (Invitrogen, Carlsbad, CA, USA) and transformed into *E.coli* C41 cells for protein expression (Lucigen Corp., Middleton, WI). Assays are currently being developed to analyze the metabolites of *R. rubrum*; first purification via chromatography column and then GC-MS analysis.

RT-PCR was performed to study the expression of A2950, compared to A2618, A2983, RrSHC and the control RpoD, a gene that's transcription is not effected by metabolic state. First, optimal binding temperatures of each primer was investigated via PCR using KAPA Polymerase, #6 *R. rubrum* gDNA template and an annealing temperature gradient of 65-75 ˚C (about 2 ˚C per well). RNA was extracted from aerobic and anaerobic cells using Ribozol extraction reagent (AMRESCO, Solon, OH, USA) and was then quantified using a NanoDrop. A two step reaction was performed with the extracted RNA to investigate the expression of gene A2950. RT-PCR was performed to produce cDNA using Promega Reverse Trancription protocol (Promega, Madison, WI, USA) and AMV Reverse Transcriptase. The resulting cDNA was treated with Rnase and then quantified with a NanoDrop. Then a PCR reaction was performed using normalized cDNA, KAPA Polymerase and optimal annealing temperatures . Gel electrophoresis was performed to analyze the gene expression. Results from two separate aerobic and anaerobic cultures were collected and will be tested for statistical significance once a third reaction is carried out.

RESULTS

R. rubrum cultures were successfully grown aerobically and aerobically for the extraction of metabolites and RNA as seen in **Figure 2**. Anaerobically-grown *R. rubrum* utilizes photosynthesis, synthesizing light-absorbing carotenoids and bacteriochlorophyll that give off a vivid red color, while aerobically- grown *R. rubrum* utilizes glycolysis and oxidative phosphorylation. Gene A2950 was cloned into a pENTR plasmid and transformed successfully into *E.coli* shown in **Figures 3 & 4.** The exact size of this gene is 1083 bp, as determined by comparison to the *R. rubrum* genome and confirmed via gel electrophoresis.

Figure 2. Anaerobically-grown *R. rubrum* (left) and aerobically- grown *R. rubrum* (right).

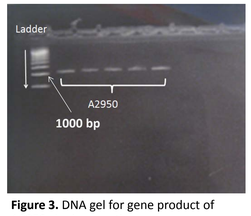

Figure 3. DNA gel for gene product of A2950 cloning reaction.

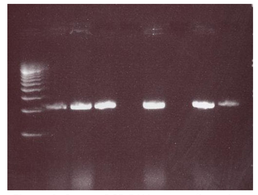

Figure 4. Colony PCR DNA gel confirmation of the transformation of pENTR plasmid containing gene A2950 in *E. coli* Top10.

The colonies with the pENTR plasmid containing gene A2950, tested in **Figure 4,** were plated on a Kanamycin plate in order to investigate metabolites produced. The attempt at growing and preserving these cells failed. Therefore the function of A2950 could not be determined at this time. The DNA from aerobically and anaerobically grown cells was extracted and utilized as a template in PCR to investigate a common optimal annealing temperature for each of the different gene primers. The optimal annealing temperature was found to be 67 ˚C (2nd well) as seen in **Figures 5-7** and was programed into the thermocycler parameters for PCR.

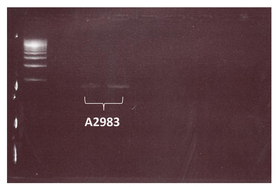

Figure 5. PCR results from optimal binding temperature from gene A2983.

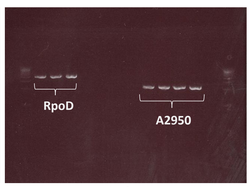

Figure 6. PCR results from optimal binding temperature from gene A2950 and Rpod

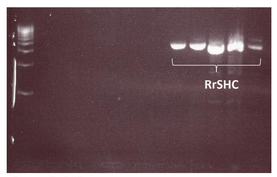

Figure 7. PCR results from optimal binding temperature from gene A2618, which did not show and RrSHC.

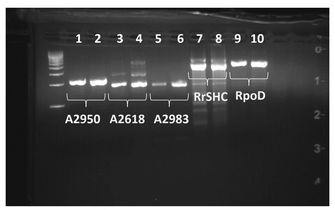

Figure 8. Results from cDNA gene expression, odd bands are anaerobic and even bands are aerobic. This illustrates that A2950 is not transcriptionally regulated.

RT-PCR was successfully carried out to investigate the expression of gene A2950, A2618, A2983 and RrSHC in aerobic and anaerobic environments, illustrated in **Figure 8**. A second RT-PCR was carried out giving similar results; gel not shown. Bands are seen in both anaerobic and aerobic lanes for all genes indicating that they are not regulated at the point of transcription.

Anaerobic and aerobic *R. rubrum* cells were extracted using hexane and ethyl acetate, then condensed and analyzed via GC-MS with squalene and lycopene standards. As seen in **Figure 10**, squalene can observed in aerobic extracts at a retention time of 18.205 minutes as compared to a squalene standards in **Figure 9.**

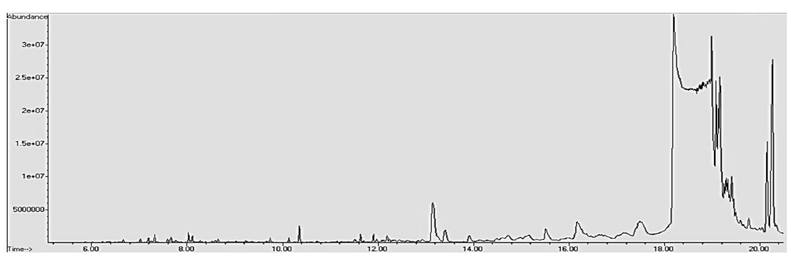

Figure 9. A chromatogram of a squalene standard produces a peak at retention time of 18.205 that was compared to aerobic extracts.

RESULTS CONTINUED

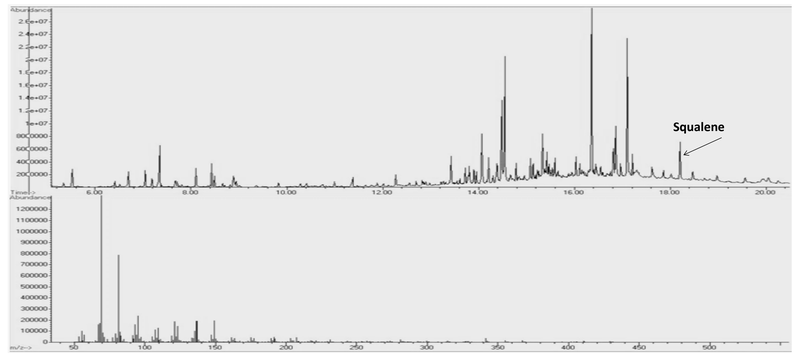

Figure 10. Squalene can be observed on the chromatogram from a *R. rubrum* aerobic hexane extract at a retention time of 18.205 as compared to standards. A molecular ion peak can be observed on the mass spectrum at m/z 410 indicating squalene.

Lycopene could not be identified in anaerobic extracts due to insignificant peaks compared to the lycopene standard. However, it is presumed that anaerobic extracts contain lycopene or similar carotenoid due to the vivid red color of the cells.

CONCLUSIONS

RT-PCR results revealed that A2950, A2618, A2983 and RrSHC are expressed in both aerobic and anaerobic environments due to the presence of bands produced in RT-PCR shown in **Figure 8**. RrSHC is a putative cyclase and is expressed in both environments as expected. A2950 is a putative squalene/phytoene synthase, therefore it makes sense that it is expressed in both environments. A2618 and A2983 are a putative aerobic intermediate synthase and a putative anaerobic intermediate synthase, respectfully, yet are expressed in both environments. This disproves the hypothesis that A2618 and A2983 are transcriptionally regulated. Considering that A2618 and A2983 are part of a differential step in terpenoid metabolism, it can be surmised that these terpenoid biosynthetic genes are post transcriptionally regulated. Statistical analysis will be carried out once a third RT-PCR is completed to determine significance. Future directions include the investigation of post transcriptional regulation in terpenoid biosynthetic genes. Further investigation of the function of A2950 and metabolite production will require successful growth and preservation of *E. coli* colonies with the pENTR plasmid containing gene A2950.

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